BIOLOGICAL AND PHYSICAL PROPERTIES OF THE HEMOTOXIN OF STREPTOCOCCI.

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The isolation of hemolytic streptococci from many different pathological lesions demonstrates their importance as pathogenic organisms. Various investigators point out two types of these organisms, one of which produces hemolysis only on blood agar, while the other produces also a hemolytic substance, or hemotoxin, in bouillon. Whether or not all laking of erythrocytes as exhibited on blood agar plates by different strains is the result of the same reaction is not clear. However, the ability to produce hemotoxin in bouillon seems to be a definite characteristic of certain strains of streptococci. Since hemolytic power is a biological function of most virulent streptococci, it is of interest to note the nature of the hemotoxin.

Frequent attempts have been made to separate hemotoxin from the organisms by filtration. The matter of filtration is of the first importance in showing whether the hemotoxin is in solution or is contained within the bacterial cells. Aronson (1902) states that hemotoxin can pass through a filter. Besredka (1901), using a Chamberland filter, noted a relation between the rate of filtration and the hemolytic strength of the filtrate and suggests that perhaps the active substance is retained in the pores of the filter. Ruediger (1903) recommends that the filter should be carefully selected, as too fine a filter removes the hemolytic property. Von Hellens (1913) and Nakayama (1919) were able to obtain hemolytic filtrates only with considerable loss of potency during the procedure. Maasen filters were used in their experiments. M'Leod (1911–12) and Braun (1912) were able to secure hemolytic filtrates by the use of Maasen filters. Lyall (1914) was not able to obtain hemolytic filtrates by the use of coarse Berkefeld filters.

Another method of separating the bacterial cells from the hemotoxin is by centrifugation. Levin (1903) reported that there was nearly 1,000 times as much hemotoxin in the sediment of a centrifuged tube of medium as in the filtrate of

the same fluid. A comparison of the sediment and the supernatant fluid after centrifugation would seem to have more value, however. Schlesinger (1903) concentrated the streptococci by centrifuging and found that hemotoxin was liberated by crushing the cells.

Little is recorded regarding the chemical nature of the hemotoxin. Ruediger (1903) states that it is composed of a toxophore and haptophore group loosely bound together. Von Hellens (1913) found hemotoxin soluble in ether and extracted it by this method. Von Lingelsheim (1912) reports that hemotoxin is of the nature of an enzyme. Lyall (1914) is of the opinion that it is not an enzyme because the hemolytic power of his cultures was destroyed by exposure to chloroform and toluene, reagents not destructive to enzymes.

The culture medium used by different workers for the production of hemotoxin varies widely. M'Leod (1911–12) found that the addition of 15 to 20 per cent of horse serum to bouillon produced the most powerful hemotoxin. Nakayama (1919) added 50 per cent or more horse serum to broth. Von Hellens (1913), on the other hand, found that the addition of horse serum in amounts of more than 40 per cent tended to decrease the amount of hemotoxin produced. Lyall (1914) used a calcium carbonate broth to which 16 per cent ascitic fluid was added.

Owing to the contradictory results reported in the literature a repetition of much of the work on this subject seemed necessary. The object of the work reported in this paper was to determine if possible something of the nature of the hemotoxin by means of a study of its filterability, the effect of adsorptive agents, the effect of centrifugation and shaking, and the basic substances from which it is produced.

In all the work a standard culture medium was used consisting of bouillon with 1 per cent of Difco peptone prepared according to the method of Huntoon (1918), to which were added 3 drops of normal horse serum per 5 cc. of the medium. The strains of streptococci used were obtained from cases of empyema and were supplied by the Army Medical School. Hemolytic tests were accomplished by adding to 0.5 cc. amounts of the proper dilutions of the test substance 0.5 cc. of 5 per cent washed rabbit corpuscles. The tubes were then incubated for 1 hour in the water bath at 37°C. The relative susceptibility of the corpuscles from different animal species was not determined. It was found that the hemotoxin of the streptococcus was not specific for red blood cells but had the power of destroying various types of muscular and glandular tissue.

Effect of Filtration and Adsorption on the Hemotoxin.

The effect of filtration on hemotoxin may be determined by a few simple experiments. It was found that there was considerable loss during passage of bouillon through Mandler filters. If the hemolytic substance is held in the pores of the filter, reversal of the filter and washing with the filtrate should restore the hemolytic titer. No hemotoxin was recovered by this method. The rate of filtration was observed to have considerable effect upon the amount of hemotoxin in the filtrate; *i.e.*, the more rapidly the bouillon was forced through the filter, the greater the amount of hemotoxin recovered. As a control on this experiment a broken filter was finely pulverized in a mortar and 2 gm. were added to each of two tubes containing 5 cc. of

TABLE I.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Untreated bouillon culture	++	++	++	++	++	+
Rapid filtration	++	++	+	−	−	−
Slow "	−	−	−	−	−	−
Reversal of the filter	−	−	−	−	−	−
Rapid adsorption	++	++	++	++	−	−
30 min. "	−	−	−	−	−	−
Addition of calcium chloride	+	−	−	−	−	−

++ designates complete hemolysis; +, partial hemolysis; −, no hemolysis.

hemolytic bouillon culture. These were shaken to insure an even distribution of the pulverized material throughout the bouillon. One was immediately centrifuged and the other allowed to stand for 30 minutes before centrifugation. The first tube showed slight diminution of hemolytic titer, while the second showed no hemotoxin present in the liquid.

The addition of calcium chloride to bouillon causes a precipitate which may act as an adsorptive agent. Barium carbonate will also remove the greater part of the hemotoxin from bouillon.

From Table I the similarity in action between rapid filtration and adsorption can be observed. It is especially significant, since the same material was used in both processes. The failure to recover hemotoxin by reversal of the filter and passage of the filtrate back

through the filter indicates either the destruction of hemotoxin or its firm combination with the filter material. If infusion bouillon is passed through a diatomaceous filter before inoculation with hemolytic streptococci good growth results but the production of hemotoxin is greatly reduced, indicating that the basic substances from which the hemotoxin is derived are likewise adsorbed, at least in part, by filtration. The fact that the addition of insoluble compounds also removes hemotoxin from bouillon seems to confirm the part played by adsorption in the process of filtration.

Effect of Centrifugation and Shaking on the Hemotoxin.

If the hemotoxin is contained in or on the bacterial cells, concentration of the cells by centrifugation should increase the hemolytic titer. A tube of hemolytic bouillon was centrifuged at high speed until the supernatant liquid was clear (about 20 minutes). Another tube of bouillon fitted with a rubber stopper was placed on a wheel revolving about 30 times a minute and the bouillon was kept in constant agitation for 20 minutes. The top half of the liquid in the centrifuged tube was removed with a pipette; part of this was used for plating on blood agar in order to determine the number of cocci present and part used for hemolytic tests. The lower half was shaken in order to get an even distribution of the sedimented streptococci and was tested in a similar manner. As a control on the presence of streptococci some of the same bouillon was treated with the pulverized filter material for 20 minutes and centrifuged for 3 minutes. The results are shown in Table II.

The counts shown in this table are of necessity inaccurate, since we have no means of knowing whether one streptococcus or a chain of several members produced the colonies. They are, however, relative and give a fair ground for comparison. The hemotoxin has evidently disappeared from the supernatant fluid, although there are some organisms present. Even though the streptococci have been concentrated to twice the number per cubic centimeter the hemolytic titer is slightly less than that of the bouillon in the control tube which had not been centrifuged. The process of shaking also reduced the titer somewhat, although the bacterial count remained the same as in the

control tube. The culture treated with kieselguhr contains enough streptococci to show some hemolysis, provided the hemolytic substance is an integral part of the cell. If a hemolytic test is set up in a hollow culture slide and the process of hemolysis studied through the microscope it soon becomes evident that hemotoxin must be free in the medium and unassociated with the bacterial cells. Red blood corpuscles are seen to assume gradually a moth-eaten appearance and finally to disintegrate, although during the whole process no streptococci have been near them. In such a test as that shown in Table II there are present 100 erythrocytes to each streptococcus. Since these organisms are non-motile it is difficult to believe that each blood cell has come in direct contact with a streptococcus. Microscopic study gives convincing evidence that such is not the case. The results of these experiments seem to indicate that the hemotoxin is not associated with the bacterial cells and that there is a mechanical factor responsible for loss of titer during centrifugation.

TABLE II.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.	No. of colonies per cc.
Untreated	++	++	++	+	−	−	1,000,000
Centrifuged, top	−	−	−	−	−	−	50,000
" bottom	++	++	+	+	−	−	∞
Shaken	++	++	+	−	−	−	1,000,000
Kieselguhr	−	−	−	−	−	−	100,000

Relation of Hydrogen Ion Concentration to the Hemotoxin.

Certain concentrations of acid and alkali are suitable reagents for causing the laking of erythrocytes. The possibility that hemolysis may be due to this cause cannot be ignored. The presence in bouillon of carbohydrates from which acid might be produced tends, however, to decrease rather than increase the amount of hemotoxin. The only apparent effect of the initial hydrogen ion reaction of the medium was to inhibit growth when the reaction was strongly acid or alkaline. Hemotoxin was obtained from bouillon having a pH value at the time of testing for hemolysis of 7.2 to 5.3. Media containing pieces of marble gave maximum hemotoxin production. Hemolytic bouillon

diluted with an equal volume of isotonic sodium carbonate solution gave results identical with those obtained by diluting to the same extent with sodium chloride solution. From these results it seems impossible that the hemolytic action can be due to the presence of an acid radical.

Relation of the Constituents of the Medium to Hemotoxin Production.

The ideal method of attacking this phase of the problem would be to withdraw one substance after another from the culture medium and test each substance alone and also the remainder of the material as to its ability to support hemotoxin production. This is not possible in all cases on account of the complexity of the constituents of

TABLE III.

Bouillon.	Growth.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Undiluted.....	Good.	++	++	++	++	++	+
Diluted to 2 volumes.....	"	++	++	++	++	++	+
" " 4 "	"	++	++	++	++	++	+
" " 8 "	"	++	++	++	++	+	+
" " 16 "	Slight.	++	++	++	+	−	−
" " 32 "	"	++	++	+	+	−	−
" " 64 "	"	++	+	+	−	−	−
Horse serum + salt solution.....	None.	−	−	−	−	−	−

the bouillon. We must constantly bear in mind the necessity of removing any bactericidal reagent from the medium and also of retaining the tonicity of the preparations at a point such that hemolysis of red blood cells will not follow from this cause.

It seemed probable that on occasions solutions would be added to the medium and it would be inconvenient and not expedient to evaporate the excess. The effect of diluting the bouillon with physiological salt solution was therefore investigated. As usual, 3 drops of horse serum were added to each tube after the dilutions were made.

From Table III it is evident that bouillon can be diluted to four times its volume before any effect on hemotoxin production is observed. Beyond this point the hemotoxin production is in inverse ratio to the dilution of the medium. In no case has the addition

of reagents approached four times the volume of the bouillon so any decrease in hemolytic titer is not due to the factor of dilution.

Relation of Phosphorus to Hemotoxin Production.—In Table I it was shown that the addition of calcium chloride to bouillon caused the formation of a precipitate. Upon analysis this proved to consist mainly of calcium phosphate, although other substances were brought down either mechanically or by adsorption. Phosphoproteins are not precipitated by this reagent. It was found, however, that streptococci during growth liberated phosphorus, presumably from the protein radicals. In order to obtain a phosphorus-free medium the following procedure was adopted. 0.6 cc. of 2.5 per cent calcium chloride solution was added to 5 cc. of bouillon and boiled. The precipitate was removed by centrifugation. The supernatant liquid

TABLE IV.

Bouillon.	Growth.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Bouillon B_1 with calcium chloride.....	Good.	++	++	++	++	+
" B_2 " " "	"	++	++	++	++	+
" B_3 " " "	"	−	−	−	−	−
" B_3 + ammonium monobasic phosphate.....	"	++	+	+	−	−
Bouillon B_3 + phosphoric acid.....	"	++	++	+	−	−
" B_3 + potassium acid phosphate....	"	++	−	−	−	−

was enriched with 3 drops of normal horse serum, inoculated with streptococci, and incubated for 24 hours. Then the process was repeated until the medium was free from phosphorus. Two or three inoculations were generally necessary. Such a phosphorus-free bouillon reinforced by 3 drops of normal horse serum supported abundant growth of streptococci but allowed production of no hemotoxin.

The addition of known phosphorus compounds to a phosphorus-free bouillon was tried. In Table IV is a tabulation of a series of such experiments. Bouillon B_1 was heated with calcium chloride once; Bouillon B_2 was heated with calcium chloride, the precipitate removed, inoculated with streptococci, incubated, and precipitated a second time. No. B_3 was carried through the process of precipitation for a third time. When chemically tested this bouillon was shown to be free from phosphorus. The amounts of phosphorus compounds

added were 0.5 cc. of 2 per cent ammonium monobasic phosphate, 0.5 cc. of 2 per cent potassium acid phosphate, and 0.5 cc. of 1 per cent phosphoric acid. These solutions are approximately isotonic for red cells as they gave no hemolysis when added to a standard cell suspension.

The results of these experiments indicate that as long as the phosphoproteins are present in the medium the maximum amount of hemotoxin is produced. The addition of phosphorus compounds restores in part but not completely the hemotoxin-producing properties of the medium. The non-production of hemotoxin in phosphorus-free bouillon is not due to the effect of the lack of buffer action, as the reaction was unchanged by the growth of the streptococci and presented a range within which hemotoxin has been produced repeatedly.

Relation of Other Chemical Factors to Hemotoxin Production.—The preceding experiment and certain others suggested the possibility that there might be present in the medium two factors or chemical essentials, one of which is able to support growth but not hemolysin production, the other supplies the necessary substances for the elaboration of hemotoxin. Experiments were instituted to separate the chemical substances contained in infusion bouillon. Metaproteins, proteoses, and peptone were isolated from the bouillon according to the methods outlined by Hawk (1916). These substances were made up to the approximate volume of the bouillon from which they were taken by the addition of physiological salt solution, and the standard amount of normal horse serum was added. Fair growth of streptococci was invariably obtained but no hemotoxin was produced. Even the mixture of all these substances in the original proportion failed to support the production of hemotoxin. It seems evident that a variety of substances may support growth but not the production of hemotoxin.

Two reagents have been used for the fractionating of bouillon, calcium chloride and ether. When calcium chloride is added to broth a heavy precipitate is formed. This precipitate can be divided by ether extraction into a waxy substance, which is perhaps cerebrin, and calcium phosphate. Neither the whole precipitate nor either of the fractions, when washed free of bouillon, supported hemotoxin production. Bouillon after removal of the calcium precipitate suffers

no loss of its hemotoxin-producing qualities. Extraction of this fraction with ether does not affect the production of hemotoxin. When the whole bouillon is extracted with ether and then precipitated with calcium chloride the fractions containing the water-soluble matter are the only ones which give hemolysis. These preparations were brought as nearly as possible to the original volume and to them was added the usual amount of serum.

If the calcium precipitate previously referred to is returned to the bouillon no effect on hemotoxin production is observed. The substance separated from the bouillon by ether extraction, whether obtained from the whole bouillon or from the calcium precipitate, when returned to bouillon considerably reduces the yield of hemotoxin. Pure cholesterol when added to the bouillon does not influence hemotoxin production. Lecithin, however, has a pronounced inhibitory action. Thus the substance which is separated from the bouillon by means of ether acts in a manner similar to lecithin. The substances in bouillon which are soluble or insoluble in ether are not essential to the production of hemotoxin of the streptococcus.

Relation of Serum to Hemotoxin Production.—When bouillon prepared with Liebig's beef extract instead of meat infusion is planted with streptococci, fair growth is obtained but no hemotoxin is produced. By using this medium as a base, opportunity is offered for determining whether the source of the hemotoxin is in the infusion bouillon or in the blood serum. To one series of tubes containing 5 cc. of Liebig's extract bouillon graduated amounts of infusion bouillon were added; to the other the same amounts of serum were added. The results of these tests are shown in Table V.

From this table it can be seen that the infusion bouillon of itself adds but little to the production of hemotoxin, whereas serum increases the production in the ratio of its presence in the medium. There is, perhaps, the requisite amount of the growth factor in either Liebig's or infusion bouillon. The basic substances for hemotoxin production are present in only a slight degree in infusion bouillon but in considerable quantity in serum. It is evident that there are certain quantitative relations between the growth factor and the hemotoxin-producing factors to be observed in the production of the maximum amount of hemotoxin. With infusion bouillon 0.05 cc., or 3 drops, of

serum are sufficient for maximum hemotoxin production, whereas with Liebig's extract bouillon 2 cc. of serum are necessary to obtain the same result. The growth factor in infusion bouillon may be diluted to four volumes as shown in Table III and still produce the maximum amount of hemotoxin. Horse serum diluted with physiological salt solution will support little if any growth.

The isolation of the hemotoxin-producing substance from serum is as difficult as from bouillon. Serum was diluted to twice its volume with salt solution and heated in boiling water until the coagulable proteins were well separated. This preparation was centrifuged until the supernatant fluid, or serum water as we have designated it, was perfectly clear. When this serum water which amounted to 5

TABLE V.

Amount of infusion bouillon + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.					Amount of horse serum + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.				
	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.		0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
cc.						*cc.*					
0.05	+	−	−	−	−	0.05	−	−	−	−	−
0.2	++	+	−	−	−	0.2	+	−	−	−	−
0.6	++	+	−	−	−	0.6	++	++	+	+	−
1.0	++	+	−	−	−	1.0	++	++	++	+	−
2.0	++	+	−	−	−	2.0	++	++	++	++	++
2 cc. of infusion + 5 cc. of salt solution.	++	++	++	++	−	2 cc. of horse serum + 5 cc. of salt solution.	−	−	−	−	−

to 10 per cent of the original serum was made up to volume and added to bouillon, hemotoxin was produced in maximum quantity. Calcium chloride solution, when added to serum water, causes a precipitate, but the hemotoxin-producing factor is but slightly diminished in the supernatant fluid. The coagulable protein and the calcium precipitate of serum water do not support hemotoxin production and growth occurs to only a slight degree.

Hemotoxin-Producing Substance in Various Organs.—The occurrence of the hemotoxin-producing substance in different organs was investigated. Water extracts of brain, liver, and kidney were prepared by infusing the macerated tissues in an equal weight of water. The meat and precipitate were removed by centrifugation and the clear

extract was sterilized by boiling for 5 minutes. These preparations were added to Liebig's extract bouillon in amounts of 0.6 cc., a quantity in the case of serum sufficient to produce a fair amount of hemotoxin. The brain extract induced little or no hemotoxin production due, evidently, to the presence of a lipoid in the medium. The liver extract produced an unusual amount of growth but no hemotoxin. This result we considered as due to the selective action of the streptococcus for the glycogen in the liver extract. The kidney extract, however, when added to Liebig's bouillon produced hemotoxin equal in amount to that elaborated in the presence of serum or serum water. These results are similar to those obtained by Kligler (1919) in his study of the growth-producing substances in tissue.

The outstanding feature of these substances is their susceptibility to alkalinity and high temperatures. Kidney infusion was distributed in 5 cc. amounts into four test-tubes. To the first tube 1 cc. of normal potassium hydroxide was added; it was boiled for 5 minutes and then exactly neutralized with normal hydrochloric acid. To the second tube were added 1 cc. of normal potassium hydroxide and 1 cc. of normal hydrochloric acid and the whole was boiled at the same time as the first. To the third tube were added 2 cc. of sodium chloride solution and the entire contents were boiled as a control when the other tubes were boiled. The fourth tube was autoclaved for 30 minutes at 15 pounds pressure. The contents of the first tube when added to Liebig's extract bouillon in any amount failed to support hemotoxin production. The contents of the second and third tubes induced hemotoxin production equal to that obtained with serum. The autoclaved infusion acted in a manner comparable to that of infusion bouillon as shown in Table V.

At first one is tempted to designate the hemotoxin-producing substance by the term vitamine, as did Kligler (1919). It is doubtful whether the word as used by the physiological chemists can be justified in this application in bacteriology. The distinction between the growth factor and the factor producing hemotoxin is marked. A variety of substances may supply the needs for metabolism. Proteoses, peptone, probably some of the amino-acids, and sugars furnish the essentials for growth. The substance essential for hemotoxin production does not support the growth of the organisms. We believe that

hemotoxin is a compound synthesized from at least two basic substances; one of these is a phosphorus compound, the other a substance of unknown composition. The latter is present in fresh tissue and body fluids, is water-soluble, and is susceptible to certain destructive processes. The hemotoxin compound is easily destroyed by mechanical and physical methods and acts as an organic compound and not as an enzyme.

CONCLUSION.

The hemotoxin of streptococcus is a labile substance affected by centrifugation or shaking. It is adsorbed by various organic and inorganic substances. Hemotoxin is produced within a wide range of hydrogen ion concentrations. It is neither in nor on the bacterial cell but is free in the culture medium. It is probably not an enzyme. There are at least two substances which are essential to the medium for the elaboration of hemotoxin, one of which is phosphorus; the other is a substance of unknown composition. The unknown component is present in small quantities in unfiltered muscle infusion, but is more abundantly supplied by blood serum and kidney infusion. This substance is not an albumin, globulin, primary or secondary proteose, metaprotein, or peptone of the medium or enriching fluid. It is water-soluble, is destroyed by boiling in alkaline solution and by prolonged heating, and is removed to a considerable extent by passage through a diatomaceous filter.

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